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(54) Title: THERAPEUTIC PROCESS FOR P. AERUGINOSA INFECTIONS USING MACROLIDE ANTIBIOTICS

(57) Abstract: Macrolides, in particular azalides such as azithromycin, are suited for the treatment of nosocomial infections caused by P. aeruginosa. The mechanism of action is the inhibition of the quorum sensing of P. aeruginosa, in particular the impediment of the las and rhl quorum sensing systems synthesis and the impediment of the synthesis of the autoinducers N-[3-oxododecanoyl]-L-homoserine lactone and N-butyrylhomoserine lactone. This allows for treatments of P. aeruginosa infections at non-inhibiting concentrations of the macrolide.

Therapeutic process for *P. aeruginosa* infections using macrolide antibiotics

Field of the invention

The present invention relates to the treatment or prevention of *Pseudomonas aeruginosa* infections.

Background of the invention

P. aeruginosa, an increasingly prevalent opportunistic human pathogen, is the most common gram negative bacterium found in nosocomial (i.e. hospital-acquired) infections. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital -acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections. Immunocompromised patients, such as neutropenic cancer and bone marrow transplant patients and particularly susceptible to opportunistic infections. In this group of patients, *P. aeruginosa* is responsible for pneumonia and septicemia with attributable deaths reaching 30%. *P. aeruginosa* is also one of the most common and lethal pathogens responsible for ventilator -associated pneumonia in intubated patients, with directly attributable death rates reaching 38%. *P. aeruginosa* bacteremia is also a source of concern in burn patients. *P. aeruginosa* outbreaks in burn units are associated with high (60%) death rates. In the expanding AIDS population, *P. aeruginosa* bacteremia is associated with 50% of deaths. Cystic fibrosis (CE) patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population.

The capacity of *P. aeruginosa* to produce such infections is due to a range of extracellular virulence factors. The secretion of some extracellular virulence factors by *P. aeruginosa* has been shown to be controlled by two complex regulatory systems, the *las* and *rhl* quorum sensing systems ("quorum sensing" is also known as "cell-to-cell-signaling"). The principles of the *las* and *rhl* quorum sensing systems in *P. Aeruginosa* have been reviewed (Van Delden, C., Iglewski, B.H., Emerging Infectious Diseases, 1998, 4(4), 551-559). The first cell-to-cell signaling system described in *P. aeruginosa* was shown to regulate expression of the virulence factor LasB elastase and was named the *las* system (Passador, L., Cook, J.M., Gambello, M.J., Rust, L., Iglewski, B.H., Science 1993, 260, 1127-1130). The *las* cell-to-cell signaling system is composed of *lasI*, the autoinducer synthase gene responsible for the synthesis of 3-oxo-C_{12}-HSL (N-[3-oxododecanoyl]-L-homoserine lactone), and the *lasR* gene that codes for a transcriptional activator protein. The second known *P. aeruginosa* cell-to-cell signaling system is named the *rhl* system because of its ability to control the production of the virulence factor rhamnolipid. This system is composed of *rhlI*, the C_4-HSL (N-butyrylhomoserine lactone) autoinducer synthase gene, and the *rhlR* gene encoding a transcriptional activator protein. This system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production (Ochsner, U.A., Fiechter, A., Reiser J., J. Biol. Chem. 1994, 269, 19787-19795). The *rhl* system is also necessary for optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, and alkaline protease.

These quorum sensing systems allows *P. aeruginosa* to delay the onset of production of virulence factors, in particularly of elastase and rhamnolipid, until their cell numbers have become large enough to overcome the body's immune system. The importance of quorum sensing in the pathogenesis of chronic infections, however, is unknown.

Prior art

P. aeruginosa is commonly combatted with antibiotics such as β-lactams, aminoglycosides or quinolones. Macrolide antibiotics, however, are not appreciated by the skilled person as useful in therapeutics or prevention of *P. aeruginosa* infections, as the minimum inhibiting concentrations (MIC's) of macrolide antibiotics for *P. aeruginosa* strains typically lie by a factor of 50-100 above the clinically *in vivo* achievable levels of macrolide antibiotics. Thus, in clinical trials of macrolide antibiotics against *P. aeruginosa* strains no effect on the viability of the microorganism was observed (e.g. for clarithromycin: Yanagihara, K., Tomono, K., Sawai, T., Kuroki, M., Kaneko, Y., Ohno, H., Higashima, Y., Miyazaki, Y., Hirakata, Y., Maesaki, S., Kadota, J., Tashiro, T., Kohno, S.; J. Antimicrob. Chemother. 2000, 46, 69-72; and Yanagihara, K., Tomono, K., Sawai, T., Hirakata, Y., Kadota, J., Koga, H., Tashiro, T., Kohno, S., Am. J. Respir. Crit. Care Med. 1997, 155, 337-342). Some studies have observed a benefit of longterm macrolide treatment in patients suffering from DPB or CF (for erythromycin: Fuji T., Kadota, K., Kawakami, K., Iida, R., Shirai, R., Kaseda, M., Kawamoto, S., Kohno, S., Thorax 1995, 50, 1246-1252; for azithromycin: Jaffe, A., Francis, M., Rosenthal, M., Bush, A., Lancet 351, p. 420).

A reduction of autoinducer production by 50 µg of erythromycin/ml has been suggested (Sofer, D.N., Gilboa-Garber, A., Belz, A., Garber, N.C., Chemotherapy 1999, 45, 335-341); the *Chromobacterium violaceum* bioassay used in this reference could only measure the C_4 -HSL autoinducer, however (McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhrabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S., Williams, P., Microbiology, 1997, 143, 3703-3711).

The need exists to provide a therapeutic process against *P. aeruginosa* infections which avoids in particularly the buildup of resistence.

Summary of the invention

One object of the present application is an improved therapeutic process against *P.aeruginosa* using macrolide antibiotics, whereby the macrolide is administered in an amount which is effective in impeding quorum sensing in the said *P. aeruginosa*. This amount will typically be appreciably below the MIC of *P. aeruginosa*. In a preferred embodiment of this object, the amount is effective in impeding *las* or *rhl* quorum sensing, and particularly the amount is effective in impeding both *las* and *rhl* quorum sensing systems. The *las* and *rhl* quorum sensing systems depend on the respective autoinducer molecules 3-oxo-C_{12}-HSL (N-[3-oxododecanoyl]-L-homoserine lactone) and C_4-HSL (N-butyrylhomoserine lactone). In a further preferred embodiment of this object, the amount of macrolide administered is therefore effective in impeding the synthesis of 3-oxo-C_{12}-HSL and/or C_4-HSL in *P. aeruginosa*.

In a particularly preferred embodiment of this object of the present invention the administered macrolide is an azalide, in particularly azithromycin.

A further object of the present invention is the use of a macrolide antibiotic for the manufacture of a medicament suited for combatting hospital-acquired *P. aeruginosa* infections, whereby the medicament contains the macrolide in an amount effective for impeding quorum sensing in *P. aeruginosa*. In preferred embodiment of this object, the medicament contains the macrolide in an amount effective to impede both *las* and *rhl* quorum sensing systems of *P. aeruginosa;* and in a particularly preferred embodiments the amount is effective for impeding the synthesis of 3-oxo-C_{12}-HSL and/or C_4-HSL in *P. aeruginosa*. Particularly preferred is the use of azalides (macrolides in which the macrolide ring is expanded by one nitrogen atom), in particularly azithromycin.

The inventors of the present application have found that macrolides, azalides and in particular azithromycin interfere with the quorum-sensing mechanism in *P. aeruginosa*. It has particularly been found that macrolides impede the *las* and/or *rhl* quorum sensing systems of *P. aeruginosa*, and that they inhibit the production of both autoinducer molecules C_4-HSL and 3-oxo-C_{12}-HSL essential to the quorum sensing systems of *P. aeruginosa*. This inhibition is achieved at concentrations much lower than the respective minimum inhibiting concentrations (MIC's) of *P. aeruginosa*.

Description of the figures

Fig. 1 a) shows the growth of *P. aeruginosa* strain PAO1 (typical experiment) and its elastase and rhamnolipid production when grown in Luria-Bertani (LB) medium in the absence (squares) or in the presence of azithromycin (circles, 2 µg / ml; upside triangles, 3 µg / ml; downside triangles, 4 µg / ml; diamonds, 5 µg / ml).

Fig. 1 b) shows the elastase activity (mean ± standard deviation of three independent experiments performed in duplicate) of supernatants of cells, grown either in the absence (squares) or the presence (circles) of 2 µg / ml of azithromycin.

Fig. 1 c) shows the expression of the *rhlAB* operon (in the *P. aeruginosa* strain PAO1 harbouring its *rhlA'-lacZ* reporter fusion, pECP60) when grown in LB medium either in the absence (squares) or the presence (circles) of 2 µg / ml of azithromycin (measured as β-Gal activity, mean ± standard deviation of three independent experiments performed in duplicate).

Fig. 2 a) shows in strain PAO1 the expression of the *lasR* and *rhlR* genes (via *lacZ* reporter fusions, measured as β-Gal activities). 1, *lasR* without azithromycin; 2, *lasR* in presence of 2 µg /ml azithromycin; 3, *lasR* in presence of 2 µg /ml azithromycin and 3-oxo-C_{12}-HSL and C_4-HSL autoinducers (10 µM each); 4, *rhlR* without azithromycin; 5, *rhlR* in presence of 2 µg / ml azithromycin; 6, *rhlR* in presence of 2 µg /ml azithromycin and 3-oxo-C_{12}-HSL and C_4-HSL autoinducers (10 µM each).

Fig. 2 b) shows in strain PAO1 the expression of the *lasI* and *rhlI* genes (via *lacZ* reporter fusions, measured as β-

Gal activities). 1, *lasI* without azithromycin; 2, *lasI* in presence of 2 µg /ml azithromycin; 3, *rhlI* without azithromycin; 4, *rhlI* in presence of 2 µg / ml azithromycin.

Fig. 3 a) shows in strain PAO1 the reduction of the production of the autoinducers 3-oxo-C_{12}-HSL and C_4-HSL. 1, 3-oxo-C_{12}-HSL without azithromycin; 2, 3-oxo-C_{12}-HSL in presence of 2 µg /ml azithromycin; 3, C_4-HSL without azithromycin; 4, C_4-HSL in presence of 2 µg / ml azithromycin.

Fig. 3 b) shows in strain PAO1 the relative expression of the *rhlAB* operon, coding for rhamnosylstransferase (measured from β-Gal activities), and the production of elastase. 1, *rhlAB* expression without azithromycin; 2, *rhlAB* expression in presence of 2 µg /ml azithromycin; 3, *rhlAB* expression in presence of 2 µg /ml azithromycin and 3-oxo-C_{12}-HSL and C_4-HSL autoinducers (10 µM each); 4, elastase production without azithromycin; 5, elastase production in presence of 2 µg / ml azithromycin; 6, elastase production in presence of 2 µg /ml azithromycin and 3-oxo-C_{12}-HSL and C_4-HSL autoinducers (10 µM each).

Detailed description of the invention

The term "subject" shall mean in the context of the present application any animal, including the mammals and man.

The term "nosocomial infections" refers in the context of the present application to infections that may rise in the said subjects when they are hospitalized. Examples of such infections are pneumonia, ventilator-associated pneumonia in intubated patients, septicemia, hospital-acquired urinary tract infections following intubation with an urinary

cathether, infections arising in immunocompromised (e.g. from neutropenia, AIDS) patients and cystic fibrosis.

The term "impeding" means in the context of the present application that quorum sensing, in particular the *las* and/or *rhl* quorum sensing systems, resp. the synthesis of the corresponding autoinducer molecules, is inhibited to an extent which is detectable by a suited assay.

The amount of macrolide which is effective for the treatment or prevention of the nosocomial *P.aeruginosa*-originated disease, by impeding quorum sensing, in particularly *las* and/or *rhl* quorum sensing and, more particularly, by impeding the synthesis of the autoinducers N-[3-oxododecanoyl]-L-homoserine lactone and/or N-butyrylhomoserine lactone in *P. aeruginosa*, will vary on the type of macrolide and on the *P. aeruginosa* strain in question and may be determined by clinical studies on laboratory animals or on human volunteers.

The primary hint that this effective *in vivo* amount was used, e.g. by administering the macrolide in the form of a pharmaceutical composition (see below), is the overcome of the nosocomial infection itself.

A further hint that this effective amount was achieved *in vivo* is the regress or absence of the symptoms associated with the *P. aeruginosa* infection, such as chronic inflammatory response or tissue damage, and which would follow the release of extracellular virulence factors by *P. aeruginosa*. It is recalled that the eventual effect of the impediment of quorum sensing by macrolides is that the population of *P. aeruginosa* keeps behaving as isolated cells

(i.e. the bacteria do not mutually perceive their presence anymore) and that this misleading prevents the population from producing extracellular virulence factors such as elastase and rhamnolipid.

Further experimental hints that this effective amount was achieved may be derived from assayed samples of the environment of use of the macrolide antibiotic within the subject (serum, plasma, sputum, tissue samples, smears), namely the site of the subject's body infected with *P. aeruginosa*. It is known that the said autoinducer molecules, essential for quorum sensing, are released by the bacteria into this environment. A comparison of samples from subjects infected with a *P. aeruginosa* strain, and not treated with macrolide, with samples from subjects infected with the same strain, but treated with macrolide, may reveal, at a given administered threshold amount of the macrolide, a statistically significant difference in autoinducer concentration between the samples from the two groups (statistically significant in consideration of the differences between the individual subjects of the groups and the variabilities in cell counts and behaviour of the bacterium strain). This threshold amount may then be considered as the "effective" amount. The samples may be assayed by any technique known in the art for this purpose. An example of an assay for the autoinducer 3-oxo-C_{12}-HSL may be the one described in Pearson, J.P., Pesci, E.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 5756-5767; and for C_4-HSL the *Chromobacterium violaceum* assay (McClean,K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R. Càmara, M., Daykin M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S.A.B., Williams, P., Microbiology 1997, 143, 3703-3711; Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E. Jr.,

Rinehart, K.L., Farand, S.K., Proc. Natl. Acad. Sci. USA, 1997, 94, 6036-6041) or the assay described in Seed, P.C., Passador, L., Iglewski, B.H., J. Bacteriol. 1995, 177, 654-659. One example of an analysed sample is the sputum of patients suffering from cystic fibrosis (Geisenberger, O., Givskov, Riedel, K., Hoiby, N., Tummler, B., Eberl. L., FEMS Microbiol. Lett. 184, 273-278; Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J., Greenberg, E.P., Nature 2000, 407, 762-764).

In particular the *in vivo* effective amount of the macrolide may be about 1 to 5 µg / ml of environment of use (e.g. serum, plasma), preferably about 1 to 3 µg /ml, and specifically about 2 µg / ml.

Exemplary macrolides that can be used in the therapeutic processes and uses according to the invention are erythromycin A and B, roxithromycin, the compound of formula (VI) of EP-B-0 699 207 and clarithromycin.

A preferred class of macrolides are the azalides, which are expanded in the macrolide ring at the C9 position by one nitrogen atom. Examples of azalides which can be used and administered according to the invention are azithromycin, the compounds (II), (III) and (IV) of EP-B-0 101 186 and the compounds (III), (V) and (VII) of EP-B-0 699 207. Particularly preferred is azithromycin.

The uses and therapeutic treatments according to the invention are suited to counteract nosocomial infections at any site within a subject's (human or animal) body which can be colonized by *P. aeruginosa* and which subsequently can develop symptoms of a nosocomial disease. Such a site may be

considered as an environment of use of the macrolide. Examples of such environments of use are the lung tracheae and bronchiae (e.g. when incised in order to introduce an intubation for artificial respiration), superficial wound lesions, and any site of introduction of a cathether into the said body (e.g. an urinary cathether), and also the entire systemic body of the patient in cases of systemic infection such as in the case of *P. aeruginosa* bacteraemia.

Examples of *P. aeruginosa* strains that can be influenced by the therapeutic process according to the invention are all strains possessing a *las* and/or a *rhl* quorum sensing system. Examples of such strains are ATCC 33347, PA B16, PA N42, PA103 and in particular the strain PAO1.

By the therapeutic process according to the invention the viability of the *P. aeruginosa* strain in question is preferably not affected by the macrolide, i.e. such treatment is non-inhibitory for *P. aeruginosa*. This type of treatment avoids the development of resistance in the *P. aeruginosa* population against the macrolide, as no selection pressure favouring macrolide-resistant strains is exerted.

The macrolides may be formulated in analogy to previously known macrolide-containing medicaments in order to carry out the processes of the invention. The amount of macrolide may be chosen such that it is effective in impeding quorum sensing in particularly the *las* and *rhl* quorum sensing systems, and specifically the synthesis of the autoinducer molecules 3-oxo-C_{12}-HSL and C_4-HSL thereof.

An example of such medicaments are oral medicaments such as tablets or capsules. It is actually only by making use of the quorum-sensing capabilities of macrolides that oral

dosage forms (which typically cannot produce more than about 1.5 µg /ml macrolide serum concentration) can be employed against *P. aeruginosa* nosocomial infections.

The oral medicaments by which the macrolides are administered may for instance be sustained release tablets and comprise, besides the macrolide, pharmaceutically acceptable excipients and diluents common in the art. These include release-retarding or release-controlling agents such as polyethylene oxide, celluloses of varying degree of etherification such as hydroxypropyl cellulose or hydroxypropylmethlycellulose, pregelatinised starch, xanthan gum, polyvinylpyrrolidone or sodium carboxymethylcellulose, diluents such as sugars (e.g. lactose or sucrose), buffering aids such mono-, di- and tribasic phosphate salts, tabletting aids such as glidants (e.g. magnesium stearate, sodium stearyl fumarate), and artificial flavours or colorants. The release properties of the tablets may be further influenced by special coatings such as for example an enteric coating. In the case of oral dosage forms the macrolide is preferably formulated as an once-a-day dosage form with a content of about 100 to 700 mg of the macrolide. This would correspond to a dosage of about 1.5 mg/kg to about 10 mg/kg of body weight per day (assuming 70 kg of patient's body weight). Preferably the content of the formulation is about 250 mg.

The oral medicament may also be a capsule comprising granules, pellets or beads of the macrolide. For the formulation of the pellets or granules themselves the same pharmaceutically acceptable adjuvants as with the tablets may be used.

In order to obtain an initial guess about the *in vivo* release of an oral macrolide formulation the in vitro release behaviour (total release vs. time) of sustained release dosage forms may be determined in a standard USP rotating paddle apparatus as disclosed in United States Pharmacopoeia XXIII (USP) Dissolution Test Chapter 711, Apparatus 2, whereby the test media may be artificial gastric or enteric juices, depending on the targeted in vivo site of release. The actually obtained *in vivo* concentrations of the macrolide in serum, plasma, sputum or different tissues are dependent on several factors such as type of macrolide, released concentration thereof in the stomach and/or intestine, rate of excretion thereof and affinity of the different *in vivo* media for the macrolide (azithromycin for instance tends to accumulate in body tissues, with rather low concentrations in serum and plasma). The determinations of *in vivo* concentrations following oral administration may be done by means of usual clinical trials using a representative panel of volunteers.

Medicaments for intravenous administration may be formulated as solutions in water, isotonic saline, isotonic dextrose or Ringer's solution. As the macrolides in their neutral form are sparingly soluble or even insoluble in water then optionally non-aqueous cosolvents such as dimethylsulfoxide, ethanol, glycerol, propylene glycol and other non-aqueous vehicles which will not interfere with the therapeutic efficiency of the preparation and are nontoxic in the volume or proportion used, may be admixed to the solution, in order to enhance the solubility of the active ingredient. Alternatively or in addition, the macrolides may be converted at the nitrogen atom of their desosamine moiety into an acid addition salt. The acids used here may be

any pharmaceutically acceptable acid such as hydrochloric, phosphoric, sulfuric, acetic, succinic, hemisuccinic (half-esterified), tartaric, hemitartaric (half-esterified) and boric acids. The ethyl hemisuccinate of erythromycin A e.g. is marketed as Erythro ES ®. In the case of the azalides conversion into a disalt is possible. The dihydrochloride of the most preferred macrolide azithromycin has been prepared e.g. in example 8 of US-A-4 474 768. Further to such pre-prepared injectable solutions, solid or pre-dissolved compositions suitable for extemporaneous preparation of solutions immediately prior to administration may advantageously be made from the macrolide. One commonly known marketed example of such a reconstitutable preparation of a macrolide is Zithromax ® (azithromycin for injection) by Pfizer. Further to the macrolide and the solvent solutions for injection or the compositions for reconstitution include liquid diluents; for example, propylene glycol, diethyl carbonate, glycerol, sorbitol, etc.; buffering agents, hyaluronidase, local anesthetics and inorganic salts to afford desirable pharmacological properties.

The concentration of the macrolide in the ready-to-use injectable solution may be such that upon use a systemic concentration of about 0,5 to 10 µg /ml serum, preferably about 2 to 5 µg / ml is attained.

The treatments of the invention impede the synthesis of the C_4-HSL and 3-oxo-C_{12}-HSL autoinducers in the *P. aeruginosa* quorum sensing system by azithromycin at concentrations well below the MIC's of *P. aeruginosa*, which in turn leads to an efficent suppression of the production of extracellular virulence factors. This opens the way for the prevention of diseases arising from these virulence factors by

treatment with macrolides. Moreover, as the 3-oxo-C_{12}-HSL autoinducer has some immunomodulatory activity in itself, stimulating the production of interleukin -8 by respiratory epithelial cells, administration of macrolides to reduce 3-oxo-C_{12}-HSL synthesis might therefore partially prevent the tissue damage arising from chronic inflammatory response in conjection with nosocomial infections.

The invention will be further illustrated by the following examples. These are merely given by way of illustration and are not meant to limit the scope of the appended claims in any way.

Examples

Example 1: Effect of azithromycin on cell growth of *P. aeruginosa*

P. aeruginosa strain PAO1 was grown for a total of 10 hours on Luria-Bertani (LB) medium containing 2, 3, 4 and 5 µg / ml of azithromycin, respectively. The cell growth in the media was measured by optical absorbance measurements (turbidity) at 660 nm in intervals of 2 h. The results are shown in figure 1 a). Exponential growth was slightly affected in the presence of 2 µg of azithromycin/ml, but no effect on the stationary growth phase was observed. Sodium dodecyl sulfate -polyacrylamide gel electrophoresis of total protein extracts of cells grown either in the absence or the presence of 2 µg of azithromycin/ml did not reveal major differences. This experiment shows that an in vitro concentration of azithromcyin of 2 µg /ml does not inhibit PAO1.

Example 2: Effect of azithromycin on elastase production

P. aeruginosa strain PAO1 was grown over a total of 16 hours in broth medium either without azithromycin or with 2 µg / ml azithromycin. Samples of the supernatants of both cultures were taken in intervals of 4 hours and the activity of elastase in the samples was determined using elastin Congo red assays (Pearson, J.P., Pesci, E.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 5756-5767). The measured elastase activity was plotted against the sampling times, giving figure 1 b). The growth curves (data not shown) were also measured by optical density measurements at 660 nm; no significant influence on the growth was observed.

Example 3: Effect of azithromycin on rhamnolipid production

P. aeruginosa strain PAO1 was grown on M9-based agar plates (Siegmund, I., Wagner, F., Biotechnol. Tech. 1991, 5, 265-268) into which a gradient of azithromycin from 0 µg/ml to 20 µg/ml was incorporated. The qualitative Rhamnolipid ® plate assay was used. The production of rhamnolipids progressively decreased with increasing azithromycin concentrations without a parallel drop in growth.

Example 4: Effect of azithromycin on the expression of the rhlAB operon

P.aeruginosa strain PAO1, harbouring the fusion gene pECP60 of rhlA' with the *lacZ* reporter (Pesci, E.C., Pearson, J.P., Seed, P.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 3127-3132), was grown for 16 hours in LB medium either in

the absence or presence of 2 µg / ml azithromycin and the activity of ß-galactosidase (ß-Gal) (Miller, J.H., "Experiments in Molecular Genetics", p. 352-355. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was measured in both cultures in intervals of 4 hours. The results of 6 experiments (mean ± SD) for each culture were plotted against sampling time (figure 1 c). The growth curves (data not shown) were also measured by optical density measurements at 660 nm; no significant influence on the growth was observed.

This experiment shows that azithromycin affects, via its interference with autoinducer synthesis, the expression of the *rhlAB* operon, coding for rhamnosyltransferase (required for rhamnolipid production).

Example 5: Effect of azithromycin on the expression of the transcriptional activator genes *lasR* and *rhlR* and on the expression of the autoinducer synthase genes *lasI* and *rhlI*.

Cultures of *P. aeruginosa* strain PAO1, harbouring the fusion gene of *lasR'* with *lacZ* reporter (pPCS10011; Pesci, E.C., Pearson, J.P., Seed, P.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 3127-3132), or the fusion gene of *rhlR'* with *lacZ* (pPCS1002; ibid.), or the fusion gene of *lasI'* with *lacZ* (pPCS223; Van Delden, C., Pesci, E.C., Pearson, J.P., Iglewski, B.H., Infect. Immun. 1998, 66, 4499-4502), or the fusion gene of *rhlI'* with *lacZ* (pLPRI; ibid.) were grown for 10 h in broth medium either in the absence or presence of 2 µg /ml azithromycin and the β-Gal activity was then determined. The results for *lasR* and *rhlR* are shown in figure 2 a) and for *lasI* and *rhlI* in figure 2 b) (plotted bars are the mean ± SD of 6 individual experiments

each). In the case of *lasR and rhlR* expression the effect of azithromycin could be almost completely compensated by co-adding to the cultures exogenous 3-oxo-C_{12}-HSL and C_4-HSL in concentrations of 10 µM each (hatched bars in figure 2 a). The co-addition of 10 µM exogenous autoinducers could not restore the expression of *lasI*.

This experiment shows that the interference of azithromycin with the autoinducer synthesis is due to its effect on the transcription of the *lasR, rhlR, lasI* and *rhlI* genes.

Example 6: Effect of azithromycin on the production of 3-oxo-C_{12}-HSL and C_4-HSL autoinducers

P. aeruginosa strain PAO1 was grown in LB medium for 12 hours either in the absence or presence of 2 µg / ml of azithromycin. The formed 3-oxo-C_{12}-HSL and C_4-HSL autoinducers were extracted from the supernatants of both cultures with ethyl acetate and their respective concentrations were measured using specific bioassays (Pearson, J.P., Pesci, E.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 3127-3132; Seed, P.C, Passador, L., Iglewski, B.H., J. Bacteriol. 1995, 177, 654-659). The results are plotted in figure 3 a). In the presence of the macrolide the concentrations of 3-oxo-C_{12} -HSL and C_4-HSL were reduced by 94 and 72%, respectively.

This experiment directly shows the interference of azithromycin with autoinducer synthesis.

Example 7: Restoration of *rhlAB* expression and elastase production by exogeneous 3-oxo-C_{12}-HSL and C_4-HSL autoinducers

P.aeruginosa strain PAO1, harbouring the fusion gene pECP60 of rhlA' with the lacZ reporter (Pesci, E.C., Pearson, J.P., Seed, P.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 3127-3132), was grown for 10 hours in LB medium either in the absence or in the presence of 2 µg / ml azithromycin, and, in the latter case, without autoinducers or with 10 mM co-added autoinducers. Both the activity of ß-galactosidase (ß-Gal) (Miller, J.H., "Experiments in Molecular Genetics", p. 352-355. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and the production of elastase, using elastin Congo red assays (Pearson, J.P., Pesci, E.C., Iglewski, B.H., J. Bacteriol. 1997, 179, 5756-5767), was measured in all three cultures after 10 h. The results are shown in figure 3 b). (plotted bars are the mean ± SD of 6 individual experiments each).

Example 8: Azithromycin film-coated tablet for oral administration

Ingredients (mg / tablet):

1) For granulate:

Azithromycin Diydrate USP (equivalent to 250 mg azithromycin)	262
Pregelatinized starch	30
Anhydrous Calcium Phosphate, Dibasic	100
Sodium croscarmellose	10
Magnesium stearate / Sodium lauryl sulfate 9:1	15

2) Coating:

Eudragit L 30 D-55 ®	20

The ingredients of 1) were wet-granulated using isopropanol as granulating fluid and compressed into tablets using an usual tabletting press. These were then film-coated with 2).

The finished tablet is suited for once-a-day, twice-a-day or thrice-a-day administration, when used in the therapeutic processes according to the invention.

Patent Claims

1. A method of treatment or prophylaxis of nosocomial *Pseudomonas aeruginosa* infections in a subject in need of such treatment or prophylaxis, comprising administering a macrolide antibiotic to said subject in an amount which is effective in impeding quorum sensing in *Pseudomonas aeruginosa*.

2. The method according to claim 1, wherein the amount is effective in impeding *las* quorum sensing.

3. The method according to claim 2, wherein the amount is effective in impeding the synthesis of the *las* quorum sensing autoinducer molecule N-[3-oxododecanoyl]-L-homoserine lactone.

4. The method according to claim 1, wherein the amount is effective in impeding *rhl* quorum sensing.

5. The method according to claim 4, wherein the amount is effective in impeding the synthesis of the *rhl* quorum sensing autoinducer molecule N-butyrylhomoserine lactone.

6. The method according to claim 1, whereby the amount is effective in impeding *las* and *rhl* quorum sensing.

7. The method according to claim 6, wherein the amount is effective in impeding the synthesis of the *las* quorum sensing autoinducer molecule N-[3-oxododecanoyl]-L-homoserine lactone and in impeding the synthesis of the *rhl* quorum sensing autoinducer molecule N-butyrylhomoserine lactone.

8. The method according to anyone of claims 1 to 7, wherein the macrolide antibiotic is an azalide.

9. The method according to claim 8, wherein the azalide is azithromycin.

10. The method according to anyone of claims 1 to 9, wherein the infection is selected from the group consisting of ventilator-associated pneumonia in intubated patients, hospital-acquired urinary tract infections, infections in immunocompromised patients, infections in patients with cystic fibrosis, septicemia, pneumonia and chronic inflammatory response in conjection with nosocomial infections.

11. The method according to anyone of claims 1 to 10, wherein the macrolide antibiotic is administered intravenously.

12. The method according to anyone of claims 1 to 10, wherein the macrolide antibiotic is administered orally.

13. Use of a macrolide antibiotic for the preparation of a medicament for the treatment or the prophylaxis of nosocomial *Pseudomonas aeruginosa* infections.

14. The use according to claim 13, whereby the medicament contains the macrolide antibiotic in an amount which is effective in impeding quorum sensing in *Pseudomonas aeruginosa*.

15. The use according to claim 14, characterized in that the amount is effective in impeding *las* quorum sensing in *Pseudomonas aeruginosa*.

16. The use according to claim 15, characterized in that the amount is effective in impeding the synthesis of the *las* quorum sensing autoinducer N-[3-oxododecanoyl]-L-homoserine lactone in *Pseudomonas aeruginosa*.

17. The use according to claim 14, characterized in that the amount is effective in impeding *rhl* quorum sensing in *Pseudomonas aeruginosa*.

18. The use according to claim 17, characterized in that the amount is effective in impeding the synthesis of

the *rhl* quorum sensing autoinducer molecule N-butyrylhomoserine lactone in *Pseudomonas aeruginosa*.

19. The use according to claim 14, characterized in that the amount is effective in impeding *las* and *rhl* quorum sensing.

20. The use according to claim 19, characterized in that the amount is effective in impeding the synthesis of the *las* quorum sensing autoinducer molecule N-[3-oxododecanoyl]-L-homoserine lactone and in impeding the synthesis of the *rhl* quorum sensing autoinducer molecule N-butyrylhomoserine lactone.

21. The use according to anyone of claims 13 to 20, characterized in that the macrolide antibiotic is an azalide.

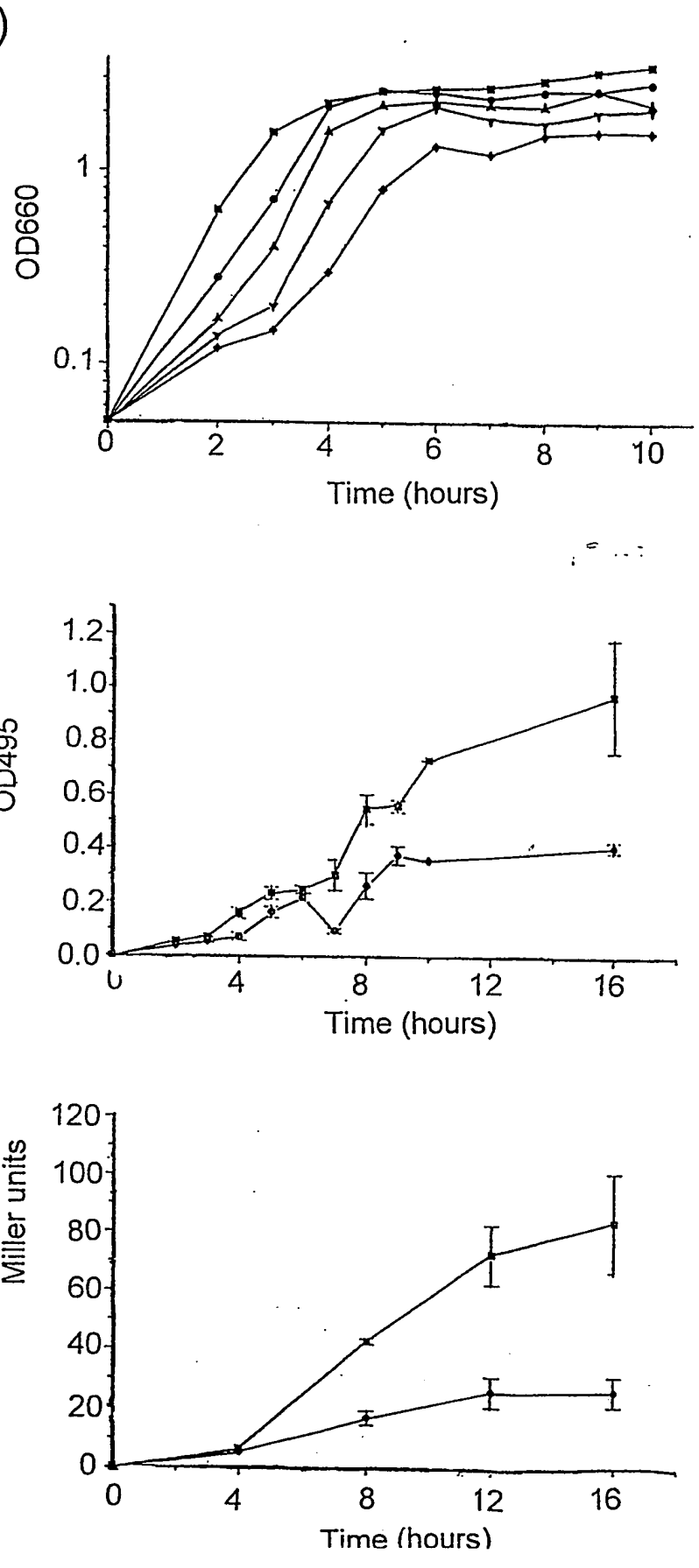
22. The use according to claim 21, characterized in that the azalide is azithromycin.

23. The use according to anyone of claims 13 to 22, characterized in that the infection is selected from ventilator-associated pneumonia in intubated patients, hospital-acquired urinary tract infections, infections in immunocompromised patients, infections in patients with cystic fibrosis, septicemia, pneumonia and chronic inflammatory response in conjection with nosocomial infections.

24. The use according to anyone of claims 13 to 23, characterized in that the medicament is suited for intravenous administration.

25. The use according to anyone of claims 13 to 23, characterized in that the medicament is suited for oral administration.

Fig. 1


a)
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0
2
4
6
8
10
Time (hours)
b)
OD495
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0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
Time (hours)
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Miller units
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4
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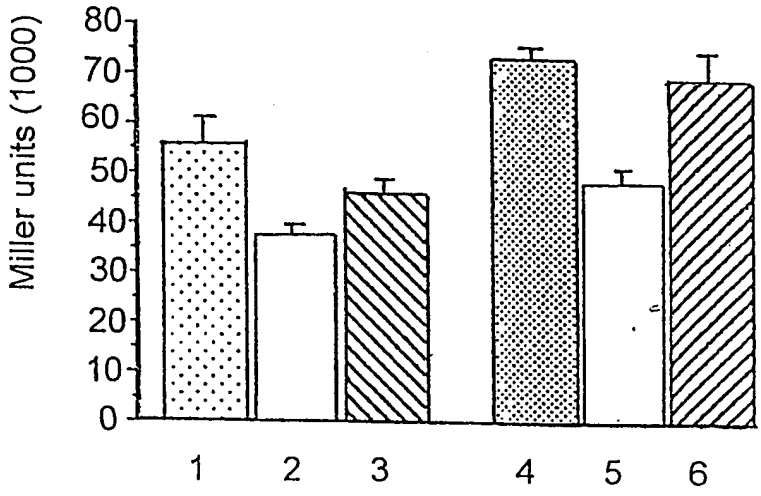
12
16
Time (hours)

Fig. 2

a)



b)

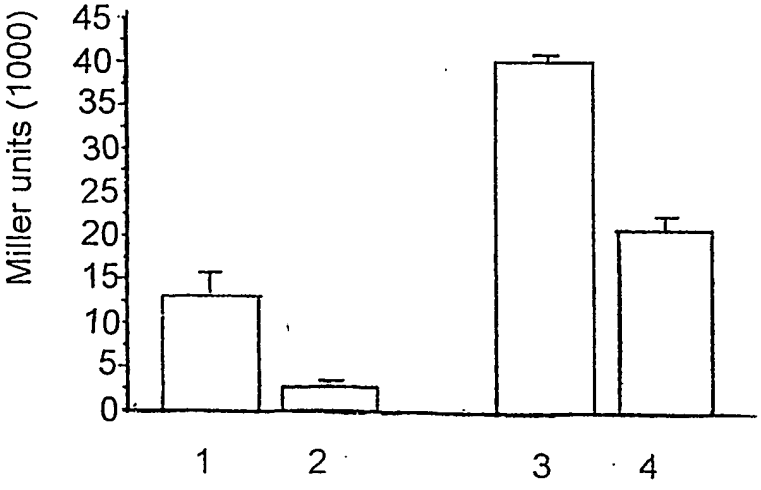
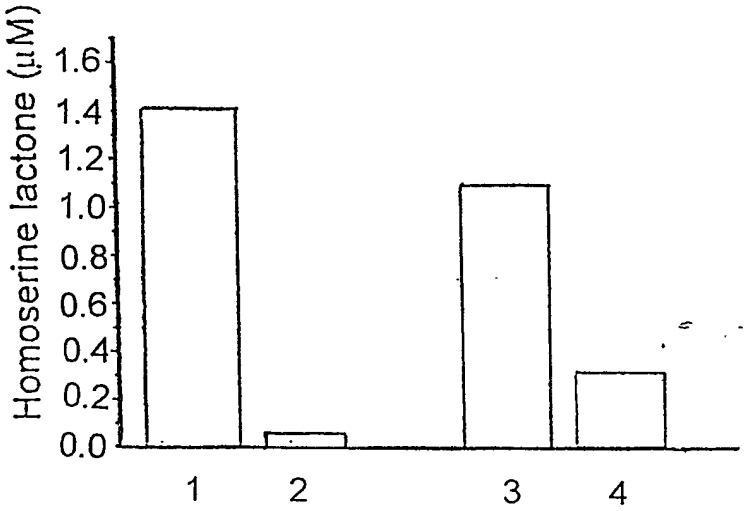
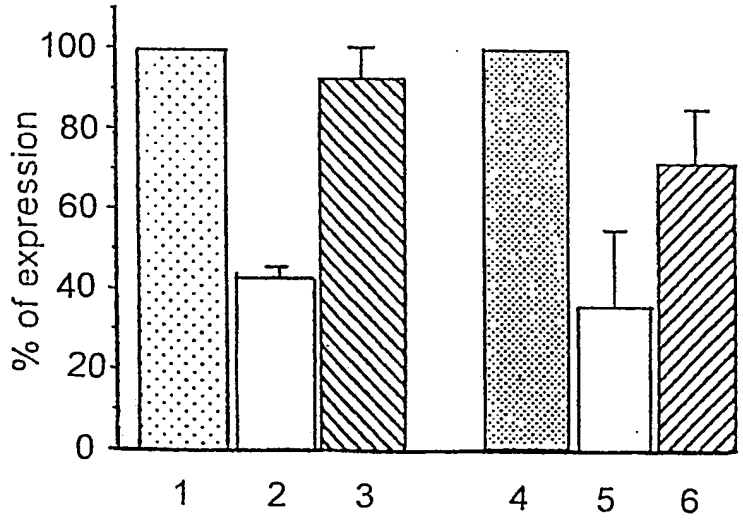


Fig. 3

a)



b)



PCT/CH 01/00532

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/7048 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SORVILLO FRANK ET AL: "Incidence and determinants of Pseudomonas aeruginosa infection among persons with HIV: Association with hospital exposure." AMERICAN JOURNAL OF INFECTION CONTROL, vol. 29, no. 2, April 2001 (2001-04), pages 79-84, XP001064885 ISSN: 0196-6553 page 79, left-hand column, paragraph 1 -right-hand column, paragraph 2 tables 1,2 page 82, left-hand column, paragraph 2 -right-hand column, paragraph 2 page 83, left-hand column, paragraphs 2,3 page 83, right-hand column, paragraph 1 --- -/--	1-25

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 April 2002	31/05/2002

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUMBAK M.J.: "VAP: Strategies for prevention and treatment." JOURNAL OF RESPIRATORY DISEASES, (2000) 21/5 (321-327). , XP001073401 page 327, middle column, line 11 -left-hand column, line 8 ---	1-25
X	JAFFE ADAM ET AL: "Long-term azithromycin may improve lung function in children with cystic fibrosis." LANCET (NORTH AMERICAN EDITION), vol. 351, no. 9100, 7 February 1998 (1998-02-07), page 420 XP002196197 ISSN: 0099-5355 cited in the application page 420, left-hand column, paragraph 4 -right-hand column, paragraph 2 ---	1-25
X	REINERT, P.: "Activity of azithromycin on Pseudomonas aeruginosa virulence factor." PATHOLOGIE BIOLOGIE, (1995) VOL. 43, NO. 6, PP. 551-553. , XP001064886 page 552, left-hand column, paragraphs 3,10 page 552, right-hand column, paragraph 3 page 553, left-hand column, paragraph 3 page 553, right-hand column, paragraph 2 ---	1-25
X	ICHIMIYA TOMOKU ET AL: "The influence of azithromycin on the biofilm formation of Pseudomonas aeruginosa in vitro." CHEMOTHERAPY, vol. 42, no. 3, 1996, pages 186-191, XP001073500 ISSN: 0009-3157 abstract page 186, left-hand column, paragraph 1 -page 187, left-hand column, paragraph 1 page 189, left-hand column, paragraph 2 -page 191, right-hand column, paragraph 1 --- -/--	1-25

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KOBAYASHI H: "Biofilm Disease: Its Clinical MAnifestation and THerapeutic possibilities of Macrolides" AMERICAN JOURNAL OF MEDICINE, XX, XX, vol. 99, no. 6, 29 December 1995 (1995-12-29), pages 26S-30S, XP002084200 ISSN: 0002-9343 page 26S, left-hand column, paragraph 1 -right-hand column, paragraph 1 page 29S, left-hand column, paragraph 1 -page 30S, right-hand column, paragraph 3-	1-25
A	TAKEOKA KAORI ET AL: "The in vitro effect of macrolides on the interaction of human polymorphonuclear leukocytes with Pseudomonas aeruginosa in biofilm." CHEMOTHERAPY, vol. 44, no. 3, May 1998 (1998-05), pages 190-197, XP001073501 ISSN: 0009-3157 abstract page 196, left-hand column, paragraph 2 -right-hand column, paragraph 2	1-25

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-8, 10-21, 23-25 relate to an extremely large number of possible compounds (macrolide antibiotics, azalides). Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds mentioned in claims 9,22 (azithromycin) and in the description at page 10, paragraphes 3-4, with due regard to the general idea underlying the present invention.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.